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ARREST OF CELL GROWTH BY NECDIN, A NUCLEAR PROTEIN EXPRESSED IN POSTMITOTIC NEURONS

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SUMMARY: Necdin is a 325 amino acid residue protein localized to the nuclei of postmitotic neurons, which withdraw permanently from the cell cycle. To examine whether necdin confers the postmitotic phenotype, necdin cDNA was stably transfected into NIH3T3 cells, in which the protein was conditionally expressed using a eukaryotic *lac* repressor-operator expression system. When the transfectants were induced to express ectopic necdin, cell growth was arrested without appreciable reduction in cell viability. The expressed necdin molecule was localized to the nuclei of the transfectants. These results suggest that necdin is a nuclear factor that governs the permanent arrest of cell growth of postmitotic neurons during development of the nervous systems of vertebrates.

Brain neurons withdraw from the cell cycle immediately after differentiation from their proliferative precursors (neuroepithelial stem cells), and enter the postmitotic state, in which the cells remain undivided throughout the lifetime. Such permanent arrest of cell division is indispensable for the maintenance of the established neuronal circuitry. Owing to this characteristic, neurons cannot regenerate even under pathological conditions where a large number of neurons degenerate and die (as in Alzheimer's

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disease for example). Therefore, the postmitotic phenotype is the most fundamental characteristic displayed by differentiated neurons. Little is known, however, about the molecular mechanism whereby neurons acquire the postmitotic state.

We have previously cloned a novel cDNA encoding a 325 amino acid residue protein, designated necdin, from a subtraction cDNA library of retinoic acid-treated P19 cells (1). Immunohistochemical studies revealed that necdin is localized to the nuclei of virtually all neurons in the mouse brain from early stages of neurogenesis until adulthood (2). Necdin mRNA is expressed in postmitotic neurons differentiated from the stem cells, whereas neither neuroepithelial stem cells nor neuronal cell lines originating from tumors such as neuroblastoma and pheochromocytoma express necdin mRNA (2), suggesting that necdin is involved in the generation and maintenance of the postmitotic phenotype. In the present study, we examined whether ectopic expression of this protein suppressed the growth of cycling cells.

MATERIALS AND METHODS

Construction of mammalian inducible vectors: The human elongation factor EF-1α promoter (3) was excised from pEF-BOS (a gift from Prof. S. Nagata) by digestion with Hind III and Xba I, inserted into Bluescript II (Stratagene), and digested with BstX I and BamH I. The fragment was directionally inserted into the operator vector pOP13CAT (Stratagene) to make pKK after removing the original promoter by digestion with BstX I and Bgl II. A 1.6 kb full-length mouse necdin cDNA fragment was excised from p4BFL (1) by EcoR I digestion, and added with Not I linker after blunting both ends. After Not I digestion, the fragment was inserted into the Not I site of pKK to make pKKNs6. pKKNs6 was transiently transfected into COS-7 cells, and expression of necdin was confirmed by immunocytochemistry using antibody C2 raised against C- terminal sequence of necdin (1).

Cloning of stable transfectants: The Lac repressor vector p3'SS (1.25 μg) (Stratagene) and pKKNs6 (1.25 μg) were stably transfected into NIH3T3 cells by the lipofectamine method recommended by Gibco BRL. After 48 h, the cells were trypsinized, plated in ten 100-mm dishes, and cultured in a selection medium containing Dulbecco's modified Eagle's medium supplemented with 10% calf serum, 400 μg/ml G418 and 250 μg/ml hygromycine. After 2-3 weeks, clones resistant to both drugs were randomly selected, and propagated in two sets of 96-well plates; one was used for screening by immunostaining with antibody C2 after treatment with the inducer isopropyl-ß-D-thiogalactoside (IPTG), and the other was kept for further propagation.

Western blot analysis: Cell lysates were analyzed by Western blotting as described previously (4): The lysates (20 μg protein for each lane) were electrophoresed on SDS-polyacrylamide gel (10% acrylamide), and transferred to the Immobilon membrane (Millipore) by electroblotting. The membrane was

incubated with antibody C2 (1:1,000) followed by the formation of avidin-biotin-peroxidase complex (Vector Labs). Immunoreactive bands were detected by the ECL western blotting detection system (Amersham).

Cell growth analyses of stable NIH3T3 transfectants: CL12, CL13, and CL19 were plated at a density of 1,000 cells per well in a 96-well plate, and incubated in 100 μl of the selection medium overnight. IPTG (final concentration, 5 mM) was added to the medium the next day (day 1), and the cells were cultured in the absence and presence of IPTG until day 5 . The cell numbers were determined by the metabolic indicator dye alamarBlue (BioSource International, Camarillo, CA), and were calculated from a standard curve which was obtained in advance using parental NIH3T3 cells. The cell numbers determined by direct counting were similar to those obtained by the colorimertric method. For analysis of growth activity by colony formation, CL12 and CL19 were plated at a density of 400-600 cells per 60-mm dish, and cultured in the selection medium for 10 days in the absence (IPTG-) and presence (IPTG+) of 5 mM IPTG. The medium was changed twice every 4 days until day 10. The cells in each colony were fixed with 3.2% formaldehyde in phosphate-buffered saline (PBS), immunostained with antibody C2 using the avidin-biotin-peroxidase technique (Vector Labs), and counted under a phase contrast microscope.

Fluorescent immunocytochemistry: The cells were fixed and stained by immunocytochemistry as described previously (5): Fixed cells were incubated with antibody C2 (1:2,000) in PBS containing 5% goat serum for 30 min at 20 °C. After washing with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulins (G+L)(1:80)(Vector Labs) for 30 min at 20°C. After washing with PBS, immunoreactive cells were visualized and photographed with a fluorescence microscope (Nikon Microphot FXA-FL).

RESULTS

We have attempted to express necdin in NIH3T3 cells utilizing a eukaryotic *lac* repressor-operator inducible expression system (6) (Fig. 1). The operator vector pKKNs6 was created in order to yield an efficient transcription of the full-length mouse necdin cDNA under the control of human EF-1α promoter (3) in the induced state. About 20 clones of transfectants containing both p3'SS and pKKNs6 were isolated in the repressed state, and three clones (CL12, CL13 and CL19) showing different degrees of necdin expression in response to IPTG were selected, and used in the following experiments. Expression levels of necdin in these clones were analyzed by Western blotting (Fig. 2A). Necdin was hardly detected in the CL12 extract, whereas CL13 and CL19 expressed high levels of necdin after exposure to IPTG; the induced level of necdin in CL19 was comparable to the level of fetal mouse brain, in which neurons are the major differentiated cell species. The

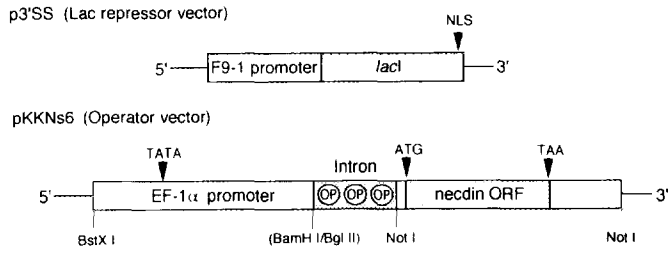

Fig.1. Expression vector constructs. p3'SS expresses *lac* repressor (*lac* I) with SV40 nuclear localization signal (NLS). The repressor protein binds to the operators (OP) present in the SV40 intron of pKKNs6 to repress the transcription of downstream necdin cDNA. When the repressor is inactivated by IPTG, transcription of necdin cDNA driven by the EF-1α promoter starts, and necdin encoded in the open reading frame (ORF) is expressed. Arrowheads: NLS, nuclear localization signal; TATA, TATA box; ATG and TAA, initiation and termination codon, respectively, for translation of necdin RNA. Restriction sites used for the construction of pKKNs6 are indicated.

molecular size of necdin expressed in CL13 and CL19 cells was 45 kDa, which was identical to that of natural necdin in the fetal brain, indicating that the open reading frame of necdin cDNA in pKKNs6 was precisely translated into the protein.

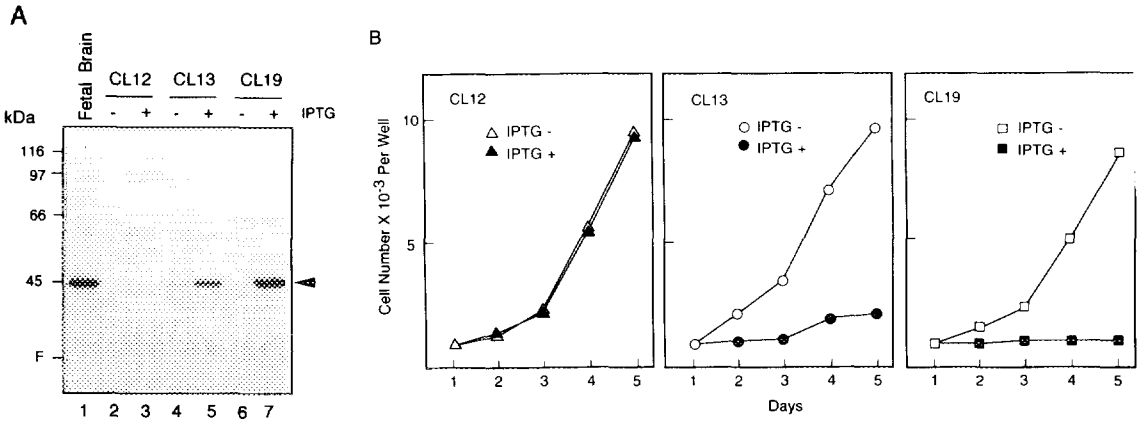

Fig.2. Ectopic expression of necdin in cDNA transfected NIH3T3 cells by use of an inducible eukaryotic expression system. A, Western blot analysis of necdin cDNA-transfected NIH3T3 cells. Cell lysates were prepared from CL12, CL13, and CL19 cultured for 24 h in the absence (-) and presence (+) of 5 mM IPTG. The fetal brain extract was prepared from an E17.5 embryo. Molecular sizes (kDa) are shown on the left. The necdin band (arrowhead) corresponds to 45 kDa. F: dye front. **B**, Growth curves of necdin cDNA-transfected NIH3T3 clones. CL12,CL13 and CL19 were cultured in the absence (IPTG-) and presence (IPTG+) of 5mM IPTG until day 5. The cell number at each time point was determined by the colorimetric method. Each value represents the mean of 4 separate experiments.

We then examined the growth activities of these transfectants cultured in the absence and presence of IPTG (Fig. 2B): IPTG had little effect on the growth of CL12, suggesting that IPTG *per se* has no adverse effect on the growth of NIH3T3 cells. In contrast, CL13 showed a marked suppression of the growth in response to IPTG (13 % of untreated control level on day 5). A more pronounced effect was noted in the high expresser CL19, whose growth was suppressed almost completely in the induced state. The degrees of growth suppression displayed by these transfectants are consistent with the induced levels of necdin in these transfectants.

In order to rule out the possibility that the growth suppression induced by necdin is attributed to its adverse effect on viability of the transfectants, we examined morphological changes of IPTG-treated CL19 cells (Fig. 3). In the induced state, CL19 cells showed a marked growth suppression (Fig.3B), but were intact without

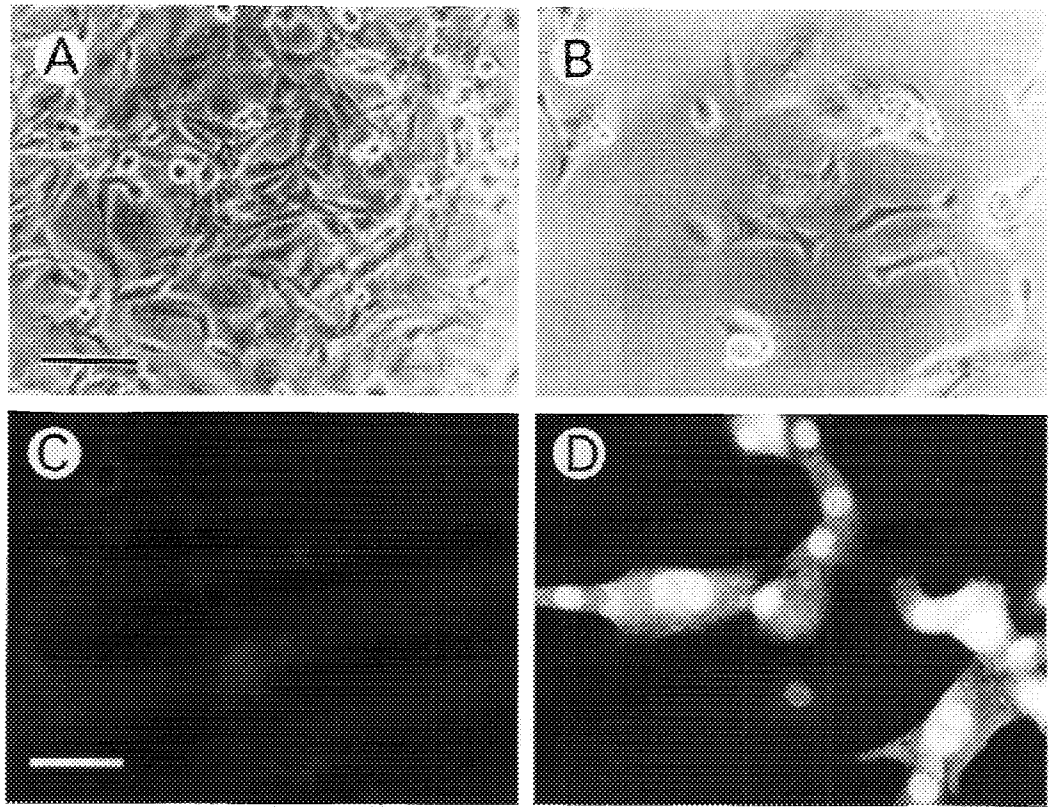

Fig.3. Phase-contrast micrographs and fluorescent micrographs of necdin cDNA-transfected NIH3T3 cells. CL19 cells were plated at a density of 3 x10^4 cells per 35-mm dish, cultured in the selection medium with and without IPTG (5mM), and morphologically examined after 3 days. **A**, **C**; untreated cells: **B,D**; IPTG-treated cells: **A,B**; phase-contrast micrographs: **C,D**; fluorescent immunocytochemistry for necdin. Scale bars, 100 μm for A, B and 50 μm for C, D.

appreciable degeneration or death. There was little difference in cell morphology between IPTG-treated CL19 cells and parental NIH3T3 cells when plated at the same cell density (not shown). The immunocytochemical analysis revealed that ectopic necdin was localized to the nuclei of IPTG-treated CL19 cells (Fig. 3D), indicating that the necdin molecule in the induced CL19 cells is properly transported into the nuclei. The cells accumulating high levels of necdin in the nuclei were morphologically intact. These findings suggest that ectopic expression of necdin has little or no adverse effect on the viability of NIH3T3 cells.

To further examine the growth suppression and viability of CL19 cells at a single cell level, we analyzed the colony forming activities of these transfectants (Fig. 4). CL12 and CL19 cells were plated at a very low density and subsequently cultured in the absence and presence of IPTG for 10 days. The distribution patterns of cell numbers in the colonies of IPTG-treated and untreated CL12 cells were similar (Fig. 4A); more than 80% of colonies contained 64 or more cells, and the frequency of colonies with 256-1024 cells (that is, 8-10 divisions for 10 days) was

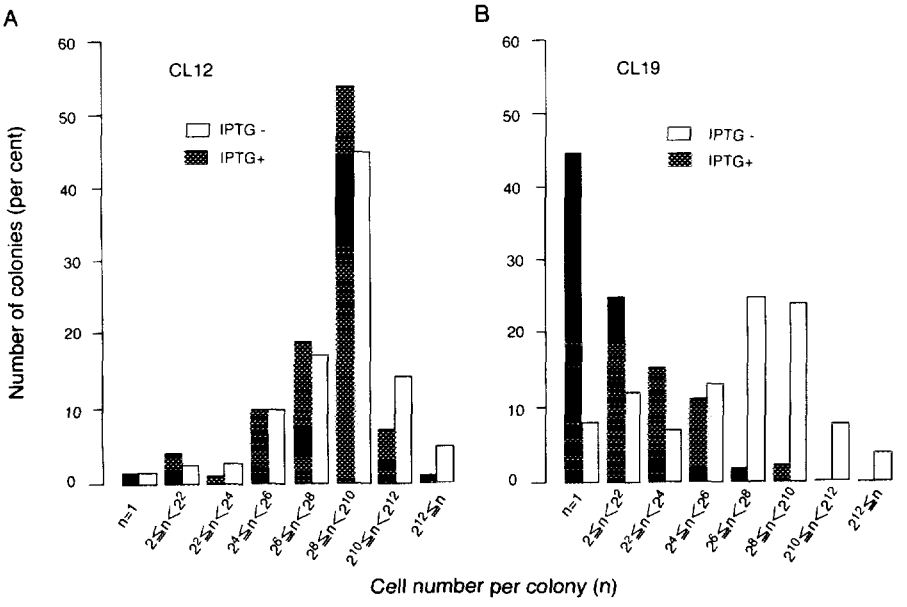

Fig. 4. Cell growth analysis of necdin cDNA-transfected NIH3T3 cells by colony forming activity. CL12 (**A**) and CL19 (**B**) were plated at a density of 400-600 cells per 60-mm dish and cultured for 10 days in the absence (IPTG-) and presence (IPTG+) of 5 mM IPTG. The cells in each colony were stained for necdin immunoreactivity and individually counted until 100 colonies per dish. The colonies were classified into 8 groups according to cell numbers. Each value represents the mean of 4 separate experiments. In both CL12 and CL19, the plating efficiencies of IPTG- treated cells were similar (about 60 %) to those of untreated cells.

the highest (Fig. 4 A). In contrast, more than 80% of the induced CL19 cells formed small colonies with less than 16 cells (that is, less than 4 divisions for 10 days) (Fig. 4B). A most striking finding was that a considerable number of the induced CL19 cells (44% of total colonies) remained single throughout the culture period. These single cells were morphologically intact, and contained high levels of necdin in their nuclei (not shown). In the repressed state, about 60 % of the colonies of CL19 cells contained 64 or more cells, but 27% of the colonies contained less than 16 cells. In such small CL19 colonies, the cells often contained necdin immunoreactivity in their nuclei (not shown), suggesting that a basal and heterogeneous expression of necdin occurs even in the repressed state in this system. These results suggest that ectopic expression of necdin in each single cell correlates with its growth suppression, and that the growth-arrested cells remain viable throughout the culture period.

DISCUSSION

The molecular mechanism whereby necdin suppresses the cell growth is not clear at present. Recently we have found that necdin binds directly to SV40 large tumor (T) antigen, a viral oncoprotein inducing transformation of the infected cells (manuscript in preparation). It is noteworthy that two major tumor suppressor gene products, p53 and the retinoblastoma susceptibility gene (Rb) protein, also form complexes with the large T antigen (7). Thus, it is likely that necdin induces the growth suppression in a fashion analogous to these tumor suppressor genes.

The necdin gene is expressed in postmitotic neurons throughout the lifetime of mouse (2). This suggests that the permanent withdrawal of differentiated neurons from the cell cycle is dependent on the constitutive and lifelong expression of necdin. Most of the neurons in the brain complete their differentiation to become postmitotic during the fetal period when the numbers of neurons in respective brain regions are primarily fixed. Therefore, the temporal and spatial patterns of growth arrest of neuronal precursors in different brain regions must be strictly controlled during development, preparing each particular brain area to acquire specific functions. It is tempting to speculate that a differential expression of the necdin gene along the neural tube causes its allometric growth that eventually gives rise to morphological and functional changes. In this regard, necdin may serve as a valuable tool to dissect the molecular basis of ontogeny and evolution of the brain in vertebrates.

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